APPENDIX - IV-A
Format for Outline (Synopsis) of Proposed Research work
(to be supplied in Four copies)
(Reference Ord. 124.11)

1. Name of Scholar (In English) - Himanshu Ojha
(In Hindi) - हिमांशु ओझा

2. Title of the Research Work :

(In English) - A study on molecular phylogeny and genetic diversity of *Citrus species* based on molecular markers

(In Hindi) - सिट्रस स्पीशीज की आणविक जातिवृतिय एवं आनुवांशिक विविधता का आणविक मार्कर्स द्वारा अध्ययन

3. Location

a. Institution/Department where the work is to be done –
Biotechnology and Molecular Biology Laboratory, Botany Department, Samrat Prithvi Raj Chauhan (S.P.R.C.) Government College, Ajmer, Rajasthan.

b. Geographical area of Investigation – *Citrus* cultivation areas of Rajasthan

4. Introduction, Importance of Proposed Investigation, Objective –

Introduction –

The genus *Citrus* L. belongs to the subtribe Citrineae, the tribe Citreae within the subfamily Aurantioideae of the Rutaceae family (Webber, 1967). The Aurantioideae is one of seven subfamilies of Rutaceae which consists of two tribes and 33 genera. Each of tribes Clauseneae and Citreae is composed of three subtribes. Clauseneae includes Micromelinae, Clauseninae and Merrillinae, and Citreae has Triphasiinae, Citrinae and Balsamocitrinae. The Citrinae is distinct from all the other subtribes in the subfamily by having pulp vesicles in the fruit. This subtribe contains three groups; primitive citrus fruit, near citrus fruit, and true citrus fruit trees. True citrus fruits have six genera: Clymenia, Eremocitrus, Microcitrus, Poncirus, Fortunella and Citrus (Swingle and Reece, 1967).
Most of genus including Citrus belongs to subfamily Aurantioideae originated from Monsoon regions and expand from West Pakistan to China, India islands, Northwest Australia, New Guinea. In this subfamily, four of 33 genus (Afraegle, Aeglopsis, Balsamocitrus and Citropsis) native to tropical Africa an one genus (Clausena) native to Monsoon and tropical Africa. Besides, Microcitrus and Eremocitrus originated from Australia (Ulubelde and Turuncgillerin,1985).
Despite its manifold economic importance and increasing demands in the global Citrus industry, Citrus taxonomy and phylogeny are controversial and confusing mainly due to the sexual compatibility between Citrus and its related genera, high frequency of bud mutations, long history of cultivation, and wide dispersion (Moore, 2001). Many of Citrus cultivars are very

closely related, apparently having diverged by mutations that alter specific horticultural traits. In addition, many Citrus cultivars produce apomictic seedlings and nucellar seedlings that differ in horticultural traits. Similarly, the level of difference in relation to species status in Citrus is uncertain. Consequently, there has been no consensus among the taxonomists as to the actual number of species that constitute the genus Citrus. In addition, taxonomic characterization leading to unambiguous identification of Citrus species and their genetic resources are essential requisites for Citrus breeding. To this end, molecular markers based on DNA sequences are being widely used in studying polymorphism between species or in populations. The application largely depends on the type of markers employed, distribution of markers in the genome, type of loci they amplify, level of polymorphism and reproducibility of products. (Fernandez et. al., 2002 and Virk et. al., 2001).

Importance of Proposed Investigation

Knowledge of genetic variation and genetic relationship among genotypes is an important consideration for classification, utilization of germplasm resources and breeding. The genetic diversity and structure of plant populations reflect the interaction of many factors, including the long-term evolutionary history of the species (e.g. shifts in distribution patterns, habitat fragmentation, and population isolation), mutation, genetic drift, gene flow and selection.

Use of morphological traits may be helpful but often inadequate in differentiation of closely related cultivars. On the other hand, certain morphologically different variants may be phylogenetically closely related. In addition morphological traits are highly influenced by the environment. Thus, using morphological traits, it can be difficult to distinguish between many Citrus cultivars. Since morphological characters are only of limited use and cytogenetical parameters are time consuming, alternate approaches, including application of appropriate molecular markers, have now been increasingly adopted to address the problems in Citrus taxonomy.

Objectives –

(i) To idendify and understand the phylogenetic relationship and systematics of the closely related species of *Citrus* using ITS sequence data of nuclear ribosomal DNA.

(ii) To determine the genetic diversity among *Citrus* species and varieties using Cytochrome P450, RAPD and ISSR molecular markers.

5. Review (Work already done on the subject of proposed Research Study)

The reviews relating to different major and minor citrus species and their characters are presented as follows.

According to cooper and Chapot (1977) the principal spesis of citrus are as follows :

Common name of *Citrus* species

Citron	*Citrus medica* L.
Sour orange	*C. aurantium* L.
Sweet Orange	*C. sinensis* Osbeck
Pummelo	*C. grandis* Osbeck
Lemon	*C. limon* (L) Burm. f.
Mandarin	*C. reticulate* Blanco
Common lime	*C. aurantifolia* Christm.
Grapefruit	*C. paradise* Macf.
Tachibana	*C. tachibana* Tan.
Indian wild orange	*C. indica* Tan.
Mauritius papeda	*C. hystrix* CD.
Melanesian Papeda	*C. macrocarpa* Mont.
Celebes papeda	*C. celebica* Koord.
Ichang papeda	*C. ichangensis* Swing
Papeda	*C. micrantha* Wester.
Khasi papeda	*C. latipes* Tan.

Risso (1813) proposed a new class represented by *Citrus limetta*. With Poieau (Risso, 1818) he established *Citrus bergamia* (the Bargamot) and *Citrus lumina* (the Sweet lemon). In 1826, he further added aurata (Poma Adami), *C. peretta, C. mellarosa* and *C. sinesis* (the Chinotto). Unfortunately many of these proposed species are now inaccessible.

Watt (1889) compiled with the works of the earlier authors on citrus classification and discussed them elaborately. He has mentioned only four species namely *C. aurantium, C. decumana, C. medica* and *C. nobills* to cover all the Indian varieties. Under *C. aurantium* he grouped 'Gajanimma' and 'Kichill' of south India which Tanaka has recognized as separate species *C. pennivesiculate* and *C. maderaspatana*, respectively.

Tanaka is most intimately associated with the citrus flora of the East. In 1933, he accepted 15 primary elements or species, for the genera *Poncirus, Fortunella* and *Citrus* of which 13 species constituted the genus C*itrus*. Later in 1937 in his further 'Revision' of Rutaceae- Aurantioideae of India and Ceylon', he enumerated as many as twenty-two different new species over his 'Primary elements'. Up to 1954 he enlarged the number considerably and accepted 144 species in the genus *Citrus* alone. He created 35 species in the mandarin group alone. Many of his new species for example *C. limettioides, C. erythorosa, C. rugulosa* and *C. Indica* are now well recognized.

Swingle (1948) simplified the citrus classification. He separated *Fortunella* and *Poniciruus* from the genus C*itrus,* under which he recognized two subgenera, 16 species and 8 botanical varieties. The notable change of botanical nomenclature accepted by him is *C. reticulata* Blanco for the mandarin, therefore, designated as *C. nobills, C. chrysocarpa, C. deliciosa*, etc. It is to be noted that under this species he included all mandarins coming under the section *Acrumen* of Tanaka. He also accepted the species *C. limon* Burm, for lemon instead of *C. limonial* Osbeck. Similarly, he considered *C. grandis* Osbeck as the correct name for Pummelo which has also been called as *C. maxima* and *C. decumana*. The confusion in limes which had been variously named as *C. acida, C. limonellus, Limonic acidissima* and *Limonic aurantifolia*, was cleared by Swingle and he gave it the name *C. aurantifolia* Swingle. It is apparent that gradually the citrus classification has become more comprehensive and elaborate. This was undoubtedly due to the fact that the

earlier workers in Europe were familiar with limited citrus plant material found in that part of the world.

Reuther *et al*. (1968) distinguished between various members of the *Citrus* spp by the differences in fruit anatomy as follows:

A.) *C. medica* [Citron] has fruit which is lemon yellow in color, large, 6-9 inches (15-23 cm) long, oblong, rough or warty, sometimes ridged, apex blunt pointed; rind thick, white except for the outer colored rim; pulp sparse; juice scant, acid, and somewhat bitter, or sweetish; juice sacs small, slender; seeds oval, plump, light colored, smooth.

B.) *C. limon* [Lemon] has fruit which ripen at all seasons; ovoid or oblong and pointed at both apex and base, about 3 inches (7.6 cm) long, either smooth or rough, light yellow in color; rind thin; flesh light colored; pulp acid, juice sacs long and pointed; seed oval, pointed at micropylar end, quite smooth.

C.) *C. aurantifolia* [Lime] has fruit which is rounded or oblong, frequently mammilate, light yellow to green; maturing irregularly throughout the year; rind thin; pulp greenish, acid; juice sacs small, slender, pointed; seeds small, oval, pointed.

D.) *C. grandis* [Pummelo] has very large fruit, globose or pyriform, light lemon to orange in color; rind very thick, white, spongy, smooth, dotted on surface with large conspicuous oil glands; segments large, commonly 10 to 14 in number, covered with thick, leathery tissue; juice vesicles large, long tapering, only loosely adhering; pulp light colored or pink, tough; flavor acid and bitter with scant sweetness; seeds monoembryonic, numerous, large, flattened or wedgeshaped.

E.) *C. paradisi* [Grapefruit] has large fruit, oblate, globose, pyriform, from out of season bloom, light lemon or orange colored; flesh grayish or pink; juice sacs large, spindle shaped, closely packed together; flavor a mingling of acid, bitterness, and sweetness or subacid; seeds polyembryonic, large, lightcolored, wedge-shaped or irregular, ridged; cotyledons white.

F.) *C. aurantium* [Sour Orange] has orange or reddish fruit, rough, slightly aromatic, bitter; pulp acid; juice sacs spindle-shaped, rather small; core hollow when fully ripe; seed flattened and wedged toward micropylar end, marked with ridge lines.

G.) *C. sinensis* [Sweet Orange] has fruit globose or oblate, light orange to reddish; rind smooth; pulp juicy, subacid; juice sacs spindle-shaped; seeds few or many, oblong-ovoid, plano-convex, generally broad, wedged or pointed at micropylar end, marked with oblique ridges.

H.) *C. reticulata* [Mandarin] has fruit flattened or depressed globose, yellow to reddish orange; segments 10-14, separating readily from one another; rind loosely attached, easily removed; seeds small, beaked; cotyledons green.

Mabberley (1997) classified edible *Citrus* into three species and four hybrid groups taking in to account recent chemotaxonomic advances.

According to Wellmer *et al.* (2004) spatially limited expression of several genes may be part of *Citrus* flower development and metabolism for model plants.

Yamamoto *et al.* (2006) determined self and cross incompatibility of 65 citrus accessions in Japan by pollen tube growth in style**.** Lemon was self compatible, whereas the six pummelos and seven out of 11 of its relatives were self-incompatible. Furthermore, among sour oranges and

their relatives, two out of six accessions were, likewise, self-incompatible as were one out of five sweet orange and their relatives.

Molecular techniques RAPD, AFLP, RFLP (Abkenar, A.A. *et al,* 2004 and Jena, S.N. *et al*, 2009), and microsatellite markers (Ghanbari, A., 2009) have been used to identify *Citrus* species with high accuracy. SSRs have been recognized as good sources of genetic markers in many plants including citrus (Kijas, J.M.H., 1997).
The existence of microsatellite sequences was first reported in citrus in 1995. Many differences among mandarin cultivars had been reported by Fang and Roose (1997).
ISSR were analyzed by Fang et al.(1998), to study phylogenetic relationships among 46 citrus L. accessions representing 35 species. Studies on development and characterization of microsetallite markers in Citrus were conducted by Ahmad et al. They concluded that microsatellite markers were able to identify cultivars at species level but individual cultivars within each species, believed to be evolved from mutation, were undistinguishable. Molecular polymorphisms among 370 mostly sexually derived citrus accessions from the collection of citrus germplasm maintained at the University of California, Riverside was detected by utilizing 24 SSR markers by Barkley *et al*.(2006). Twenty four microsatellite loci on 12 genotypes of Citrus, Poncirus, and an intergeneric hybrid were evaluated by Yaly *et al*. SRAP markers were studied by Uzun *et al.*(2011), to evaluate genetic diversity among 27 grapefruit (C. paradisi Macf.), 5 pummelo (C. maxima (Burm.) Merr.) accessions and 4 pummelo hybrids.

Molecular differentiation in 24 accessions representing 19 taxa of Indian citrus through sequence analysis of ITS region of nrDNA (nuclear ribosomal DNA) were studied by Kumar *et al.*(2013). First genome, based exclusively on Sanger sequencing, is from a haploid plant derived from Clementine‘ mandarin, to serve as the reference genome for citrus (Gmitter, J.F.G.,*et al.* 2012). Characterization was done in 65 mandarin accessions by using simple sequence repeat (SSR-14) and sequence-related amplified polymorphism (SRAP-21) based molecular approaches by Kacar *et al*(2013).

ISSR marker for identification of zygotic and nucellar seedlings in citrus interploid crosses were examined by Tusa *et al.*(2002). RAPD and Expressed Sequence Tag (EST)-SSR markers were used to characterize the zygotic and nucellar seedlings after introgression crosses of mandarin (C. reticulata) and pummelo (C.maxima) by Rao *et al.*(2008).

In *Citrus*, a wide variety of DNA based markers has been used in order to study their genetic variation as well as phylogenic and taxonomic relationship among different genera, and some of the important examples are: Microsatellite (Kijas, J.M.H. *et al.;* 1995 and Pang, X. *et al*; 2003), RAPD (*Federici, C.T. et al; 1998 and* Nicolosi, E. *et al.* 2000) RFLP *(Federici, C.T. et al; 1998)*, ISSR (Fang and Roose; 1998) organelle genome analysis, PCR–RFLP analysis of non-coding regions of chloroplast DNA (cpDNA) and sequence analysis of cpDNA region, and sequence data analysis of ITS region of nrDNA (Kyndt, T. *et al*; 2010; Pessina, D. *et al;* 2011; Xu, C.J. *et al.* 2006). These molecular studies have provided some insight to *Citrus* phylogeny and three species concept was generally supported.
Pessina *et al*(2011)., investigated the fingerprinting and phenotyping of 54 distinct accessions, including 43 genotypes of the *Citrus* species (18 species or supposed species) and 11 genotypes of the Poncirus genus by using RAPD markers. The results of the multidisciplinary analyses confirmed a remarkable differentiation between *Poncirus* and *Citrus* genera and highlighted a

close relationship among the three investigated Citrus species but a distinct difference between these three species and other species in the Citrus genus. RAPD fingerprints pointed out a variation gradient between *C. limon* and *C. medica*, with *C. limonimedica* as a possible intermediate species.

Gouri Sankar *et a*l.(2014), used RAPD markers to evaluate genetic similarity and inter relationship among twelve sweet orange varieties. They reported that Jaffa and Kodur Sathgudi were genetically closer with value 0.84 followed by Himakuntla Sweet orange and Kodur Sathgudi (0.80). Sathgudi Tirupati and Ankalamma Gudur Sathgudi formed one cluster and remaining varieties formed another cluster which in turn divided into two sub-clusters were Nadimpalli Sathgudi and Valentia formed first sub-cluster and Mosambi and Red blood Malta formed second subcluster; Jaffa and Kodur Sathgudi formed as one group and Ananthapur Sathgudi, Himakuntla Sweet orange, Valentia late and Hamlin Sweet orange did not resemble any other variety.

Simple sequence repeats (SSRs) or microsatellites are short sequence elements composed of tandem repeat units one to seven base pairs (bp) in length (Tautz, D.; 1989). SSRs are becoming increasingly widespread because it is co-dominant, multi allelic, highly polymorphic genetic markers and appropriate for genetic diversity studies, evenly distributed throughout the genome and regarded to be the most reliable marker (Goldstein, D.B. et al, 1999).

Amar *et al.*(2011), studied the genetic diversity among 24 Citrus and its relative species by using 61SSRmarkers to evaluate the level of polymorphism and discriminating capacity. In their study, a total of 596 polymorphic amplicons were observed in SSR markers with average polymorphism information content (PIC) of 0.97. High levels of polymorphism were recorded for SSR. The highest correlations (r = 0.930) were obtained between SSR and SRAP markers, whereas SSR and CAPS-SNP were poorly correlated (r = 0.833). Cluster analysis was performed to construct dendrograms using unweighted pair group method arithmetic average (UPGMA). The dendrogram from SSR data was most congruent with the general dendrogram.

Shrestha *et al*.(2012), studied the genetic diversity of 62 acid lime landraces, using SSR markers. Twelve SSR primer pairs were used to assess the genetic diversity of acid lime. The average genetic similarity level among the 62 accessions was 0.77, ranging from 0.54 to 1.0 and separated five major cluster groups. Total of 33 alleles were detected by eleven primer pairs and size of alleles ranged from 50 to 225. Average polymorphic information content (PIC) value was 0.50, whereas highest 0.75 and lowest 0.18 was observed in CAT01 and GT03 loci respectively.

Ahmed S. *et al.* (2017) studied genetic diversity in *Citrus* fruit crop by molecular markers and determined that cpDNA is especially useful in phylogenetic analyses of *Citrus* due to its evolutionary conservatism, relative abundance in plant tissue, small size and pre dominant uniparental inheritance.

ITS region is an interesting subject for evolutionary and phylogenetic investigations (Baldwin et a1., 1995) as well as biogeographic investigations (Baldwin, 1993). The sequence data of the ITS region has also been studied earlier to assess genetic diversity in cultivated barley (Petersen & Seberg 1996). Generally, the ITS has become an important nuclear locus for systematic molecular investigations of closely related taxa. This is because the ITS region is highly conserved intra- specifically, but variable between different species (Bruns et al., 1991; Hillis & Dixon, 1991). Furthermore, the ITS region evolves much more rapidly than other conserved regions of rDNA (Baldwin et al., 1995). Thus, phylogenetic studies based on nrDNA, ITS sequences have provided novel insights into plant evolution and hybridization in various plant species (Sang et al., 1995; Wendel et al., 1995; Buckler & Holtsfoid 1996; Quijada et al., 1998;

Semerikov & fascoux, 2003). The ITS region and tmL intron are the most widely used markers in phylogenetic analyses of the Brassicaceae (Koch et al., 2003a; Koch et al., 2003b). The ITS sequences have been successfully used in studying phylogenetic and genomic relationships of plants at lower taxonomic levels (Baldwin et al., 1995; Wendel et al., 1995).

6. Research Gaps identified in the proposed field of investigation. (Based on the review)

The taxonomy of *Citrus* is highly controversial and confusing due to the sexual compatibility between *Citrus* species and its related genera, high frequency of bud mutations, apomixis (adventive nuclear polyembryony), long history of cultivations and wide dispersion (Due to the enormous variability in *Citrus*, it leads to the presence of diverse cultivars within a species. Several such unidentified variants or probable natural hybrids of *Citrus* still exist in India with no taxonomic identity. Several of these variants lives are established local cultivars and used for various economic purposes. Bases on the morphological characterization, identification of these unknown variants of *Citrus* is very difficult due to plasticity in the morphological characters. In such cases, molecular markers, particularly DNA-based markers, i.e., Simple sequence Repeat (SSR) or microsatellites (Hamada and kakunaga, 1982, Tautz and Renz, 1984), Inter Simple Sequence Repeat (ISSR) (ziekiewicz et al. 1994), Amplified Fragment Length Polymorphism (AFLP) (Vass et al. 1995), RAPD etc. are highly useful for understanding level of genetic diversity, identification of closely related varieties/hybrids/ cultivars, inferring systematics, phylogeny, management and improvement of crops.

Knowledge of the levels and distribution of genetic diversity are important for designing conservation strategies for threatened and endangered species. Preservation of the genetic diversity represented in all the plant ecosystems throughout the world has become a major issue of international concern. The loss of increasingly large numbers of plant species through habitat destruction threatens the availability of a diverse plant germplasm base which will be needed to feed future generations. Advances in biotechnology, especially in the area of in vitro culture techniques and molecular biology provide some important tools for improved conservation and management of plant genetic resources.

The present study highlights the usage of different marker system for studying genetic diversity across DNA level in the genus Citrus. All the marker techniques provided useful information on the level of polymorphism and genetic diversity in Citrus, showing their utility in the characterization of germplasm accession. RAPD markers, as a fast and simple technique, can detect enough polymorphism to differentiate between different Citrus species and cultivars and to understand their inter-relationships. RAPD markers are useful for the construction of a linkage map because a sufficient number of markers can be generated and used for construction in a relatively short period of time. RAPD markers are more stable since more than 50 % of the polymorphisms observed showed linkage and mapped to a specific linkage group. Although, RAPD provides better results in some contexts as compared to other types, but RAPD had problem of reproducibility and transferability among the laboratories. On the other hand, ISSR markers as a powerful tool can differentiate closely related individuals of Citrus. The relationships of unknown genotypes of Citrus with known varieties can also be clarified. SRAP markers could be more advantageous over SSR markers due to occasional loss of amplification sites of SSR primers in distant Citrus relatives and its relative simplicity. The SSR may be more useful for segregation studies and genome mapping in Citrus. All these kind of markers have potential use in studies of diversity, linkage mapping, cultivar identification, and germplasm

organization. As a result the use of retrotransposon based markers can be a valuable tool for Citrus breeders. In summary, the combination of different kinds of molecular markers proved to be a powerful tool in carrying out a more complete analysis of Citrus phylogeny and origin.
Sampling of wild and semi wild species of Citrus, which are not yet studied would clarify the probable speciation and sequence of founding events that gave rise to this species, and possibly other relatives. This could be achieved by a combined approach of phylogeography and classical population genetics using other molecular markers, and perhaps DNA sequencing data, which could be used to identify the population phylogenetic histories. The second promising line of investigation would be to determine how crossing may have affected the population structure of this species. It would also be interesting to determine how utilization by the local people has influenced the structure of genetic diversity of this species. The last important line of research would be to study the mechanisms of pollination, seed and clone dispersal, as well as the variation in breeding system for this species. An integrated research program that combines genetic analyses with studies of reproductive biology may provide further valuable data that would greatly extend the present conclusions.

7. Major Hypothesis.

Presently, taxonomic classification of *Citrus* species and varieties and their genetic relationships is highly ambiguous and confusing, therefore, through this investigation we hypothesize that results of present investigation envisage more clarity in the genetic relationship among the different species and their varieties at molecular level using appropriate tools and techniques of molecular biology.

8. Research methodology.

DNA Extraction

Genomic DNA will be extracted following the Cetyl Trimethyl Ammonium Bromide (CTAB) protocol described by Saghai-Maroof ***et al.*** (1984) with minor modifications. Leaf tissue (1 g) will be ground to fine powder in liquid nitrogen and transferred to 10 ml 2 X CTAB buffer containing 100 mM Tris (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, 2% CTAB (w/v) and 10 mM 3-mercaptoethanol (added freshly). It will be mixed vigorously by vortexing and incubated at 60°C for 30 min followed by treatment with equal volume of chloroform: isoamyl alcohol (24: 1). The upper phase, obtained by centrifugation at 10,000 rpm for 15 min at room temperature, will be transferred to a fresh autoclaved centrifuge tube and o it will be added 1/10 volume 3M sodium acetate (pH 5.2) and 1/2 volume 5M NaCl. DNA will be precipitated using 0.6 volume chilled isopropanol and pelleted bμy centrifugation at 10,000 rpm for 10 min at 4°C. The supernatant will be decanted and the DNA pellet will be washed twice with 70% ethanol. DNA pellet will be air dried and suspended in 500 pi of 1 X TE (Tris EDTA).

DNA Quantification

DNA concentration will be estimated using spectrophotometric method. Absorbance of the solution will be measured at wavelengths 260 nm and 280 nm. The DNA concentrations will be calculated using following formula:

$$DNA\ concentration\ (\mu g / ml) = \frac{50 \times OD_{260} \times\ Dilution\ Factor}{1000}$$

The ratio of OD_{260}: OD_{280} will be calculated. The same procedure will be followed for quantifying all the samples. DNA samples will be diluted to final concentration of 5ng/µl for use in PCR analysis. DNA samples which will have best quality as evident on agarose gel and an OD_{260}/OD_{280} ratio nearer to 1.8 will be used for further analysis.

Agarose gel preparation

For visualization of genomic DNA 0.8% and for amplified products 1.5% agarose gels will be prepared. Agarose powder will be added to 1 X TAE buffer and boiled in microwave oven. This solution will be cooled to 45-50°C under running tap water. To it 0.5 µg/ml ethidium bromide (EtBr) will be added, shaken gently and poured into gel casting tray having pre-adjusted comb. The gel will be allowed to set at room temperature.

Electrophoresis

The comb will be carefully removed and the gel casting tray will be placed in a submarine gel tank. The gel tank will be then filled with 1 X TAE buffer ensuring that the gel will be completely submerged in the buffer (Ranade *et al.* 2006). Samples will be prepared by taking 4.0 µl of DNA and 1.0 µl of 10 X bromophenol blue dye (0.25% Bromophenol Blue and 50% glycerol) on a Parafilm™ strip and mixed well with the help of a micropipette. PCR products will be electrophoresed in 1 X TAE buffer for ca. 3 h at 80 V on a 1.5% agarose gel matrix. All the amplified products will be separated on 1.5% agarose (w/v) gel, stained with 0.5 µl/ml ethidium bromide. 1 Kb and 100 bp DNA ladder (Bangalore Genei, India) will be mixed and used as molecular weight marker for comparison of amplified products. Gels will be visualized and photographed under UV light using a Gel Documentation System from "Biovis" (Expert | Vision, Mumbai, India). All reactions will be repeated thrice to confirm the results.

ITS Sequencing -

The internal transcribed spacers ITS l and ITS 2 will be amplified with different sets of primers. ITS 1 with 5.8S-coding regions will be amplified by primers ITS 1 (forward) and ITS 2 (reverse) whereas ITS 2 with 5.8S-coding regions will be amplified with primers ITS 3 (forward) and ITS 4 (reverse).

The forward and reverse combinations of primers ITS l x ITS 2 and ITS 3 x ITS 4 will be used in a final volume of 25 µl containing 50 ng of template DNA, 2.5 µl of 10X assay buffer, 25mM (2.0 µl) $MgCl_2$, 200µM (0.5 µl) dNTPs mix (10 mM stock), 2.0 µl (20 pmol) primer (10 pmol/µl stock), and 1.0 unit (0.2 µl) of Taq DNA polymerase (5U/ µl stock).

All the reagents of the reaction mixture will procured from Bangalore Genei Pvt. Ltd., Bangalore, India, except primers. The ITS amplifications will be performed in a thermal cycler (BIO-RAD T100, USA).

First cycle consisted of initial denaturation of template DNA at 94°C for 04 minutes, followed by 35 cycles of denaturation at 94°C for 01 minute, primer annealing at the melting temperature Tm°C, ranging between 50.4°C to 58.8°C as mentioned by the supplier for 01 minute, primer extension at 72℃ for 02 minutes; and final extension at 72℃ for 10 minutes. Amplified products will be resolved by electrophoresis on 1.5% agarose gels prepared with 1 X TAE (Tris-acetate-

EDTA) buffer system, run at 5 V/cm, and stained with ethidium bromide solution (0.5 μg L^{-1}). A 100 bp DNA ladder (Invitrogen, Bangalore, India) will be used to estimate the molecular weights of the amplified products.

Purification of PCR **product**

The twelve amplicons (ITS 1-2 primer coded) will be taken and subjected to PCR purification. Since the amount of material will be approximately 20μl, this will mixed with 100 μl of PB buffer; also 100μl of amplified cDNA and 500μl of PB buffer will be added to it in a ratio of 1:5. The solution will then transferred to a spin column and centrifuged at 13,000 rpm at 25°C (RT) for 1 minute. Further the solution will emptied in the collection tube and placed below the spin column in to which 750 μl of PE buffer will be added, this will again centrifuged at 13,000 rpm at 25°C (RT) for 1 minute. Once again the solution will emptied in another collection tube and placed below the spin column and centrifuged at 13,000 rpm at 25"C (RT) (Dry Spin) for 1 minute. 50 μl of EB buffer will then added at the centre of the spin column and then finally once again centrifuged at 13,000 rpm at 25°C (RT) for 1 minute.

To each of the PCR tube (sample which had been cycle sequenced), 10 μl of DI water will be added. Further, the following steps will performed. 54 μl of this Master Mix will transferred into each tube and incubated at room temperature for 15 minutes. The samples are then transferred in the eppendorfs labelled for each sample. These will then spin at 10,600 rpm for 30 minutes at room temperature (22°C).The supernatant will be discarded carefully and 100 μl of 70% ethanol added to the contents will left in the eppendorf. Once again the mix obtained will spin at 10,600 rpm for 5 minutes at room temperature. These 2 steps will be repeat again. Finally the supernatant obtained will discarded, the contents of the eppendorf tube will be air dried for 15 minutes. 20 μl of Hi-Di formamide will then added, and the tubes will vortexed and briefly spin, and later transferred to the 96 well- plate. The samples will be subjected to heat shock in the thermal cycler at 95°C for 2 minutes, and then keep at 4°C for some time. 20 μl Hi-Di formamide will be added in rest of wells and placed in the DNA sequencer machine.

Identification of putative Cytochrome P450 from the *Citrus* EST database.

Using the pyrosequencing method, putative P450 sequences from *Citrus species* will be collected from a ginseng EST database and will be assembled into large contigs. Each representative P450 member will be used as a query sequence and search against the BLAST P search process conducted at the amino acid level. Each preferentially expressed member with an identified family will be used to predict the family of putative P450 genes of *Citrus species*. For each query, BLAST P hits will be collected in a file when the deduced amino acid sequences will be more than 40% identical to the corresponding query sequence. In this way all putative P450 sequences will named according to the standard system of P450 nomenclature. These assigned families were recorded and used for further analysis.

Generation of the unigene dataset.

The unigene dataset contains a set of non_redundant sequences composed of singlets and contigs. Generating a unigene dataset requires several steps to process the sequences. All are integrated and freely accessible online. Data will be uploaded to the server using the default

parameters. The bioin_ formatics pipeline starts with sequence cleaning, masking of low quality sequence, removal of repetitive elements, and organelle sequences masking. The resulting sequences will be used to generate contig sequences using the CAP3 software.

Subcellular localization and phylogenetic analysis of putative Cytochrome P450s.

Partial or full_length EST sequence at amino acid level will be used to predict the putative subcellular localization of cytochrome P450. Predictions of subcellular localization will be fulfilled using the Target P program with default parameters, which are available at the center for Biological Sequence Analysis. A family member of predicted P450 sequence and representative member of the known P450 families from other plants will be used for the construction of a phylogenetic tree. The query sequence will aligned to known P450 sequences from other plants, using the ClustalX program. The aligned sequences will be used to build a phylogenetic tree following the neighbor_joining method (NJ) using Mega 4 software. The significance level of the NJ will be examined by bootstrap testing with 1000 repeats. The tree will be represented using Boot strap view.

9. List of references cited.

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Signature of the Candidate with date

Outline Approved

Name and Signature of supervisor with date & seal.